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(56) Documents Cited
EP 0617966 A1 WO 92/04913 A1 WO 91/12334 A1
WO 89/11862 A1 US 5428011 A US 5413989 A
US 5216004 A US 5102868 A US 5071834 A
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(54) Pharmaceutical comprising a stimulator of activin and/or inhibin

(57) A pharmaceutical for the treatment of fibrotic disorders and the promotion of wound healing (with reduced scarring) comprises a stimulator of activin and/or inhibin. The activator may be activin and/or inhibin or a fragment or analogue thereof, an inhibitor of activin metabolism, a stimulator of activin synthesis, or an antagonist of an antagonist of activin and/inhibin (preferably an antagonist of follistatin).

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Pharmaceutical Composition

The present invention concerns pharmaceutical preparations for promoting the healing of wounds or fibrotic disorders, in particular for promoting the healing of wounds or fibrotic disorders with reduced scarring, and for promoting the healing of chronic wounds.

By "wounds or fibrotic disorders" is meant any condition which may result in the formation of scar tissue. In particular, this includes the healing of skin wounds, the repair of tendon damage, the healing of crush injuries, the healing of central nervous system (CNS) injuries, conditions which result in the formation of scar tissue in the CNS, scar tissue formation resulting from strokes, and tissue adhesion, for example, as a result of injury or surgery (this may apply to e.g. tendon healing and abdominal strictures and adhesions). Examples of fibrotic disorders include pulmonary fibrosis, glomerulonephritis, cirrhosis of the liver, proliferative vitreoretinopathy, repair following myocardial infarction, including myocardial hibernation.

In particular, there is a lack of compositions for promoting the healing of wounds or fibrotic disorders with reduced scarring. Scar tissue formation, although providing mechanical strength to a healed wound, can be unsightly and may impair the function of the tissue.

This is particularly the case in wounds which result in scar tissue formation in the CNS, the scar tissue inhibiting the reconnection of severed or re-growing nerve ends, so significantly affecting their function.

There is also a lack of compositions for use in the treatment of chronic wounds, for example venous ulcers, diabetic ulcers and bed sores (decubitus ulcers), especially in the elderly and wheel chair bound patients. Such compositions may be extremely useful in patients where wound healing is either slow or in whom the wound healing process has not yet started. Such compositions may be used to "kick-start" wound healing and may then be used in combination with compositions (e.g. those of PCT/GB93/00586) which promote the healing of wounds or fibrotic disorders with reduced scarring. Hence not only may a chronic wound be healed, but it may be healed with reduced scarring.

According to the present invention there is provided a composition for promoting the healing of wounds and fibrotic disorders with reduced scarring comprising a stimulator of Activin and/or Inhibin

By 'stimulator' is meant anything which may stimulate the quantity or efficacy of active Activin and/or active Inhibin at a site. This may be Activin or Inhibin itself or a fragment or an analogue thereof. An analogue may, for example, have a longer half-life than Activin or Inhibin, or it may have a different binding affinity for its receptors. A fragment may comprise at least that part of Activin or Inhibin which is required to allow it to stimulate its receptors. Alternatively, it may, for example, be an inhibitor of Activin metabolism, or it may be a stimulator of Activin synthesis. For

example, it may be an analogue of a fragment of activin or inhibin which is bound by a degradative enzyme. It may be a mimotope (Geysen, H.M. *et al.*, 1987, Journal of Immunological Methods, 102: 259-274) made to a fragment of Activin or Inhibin which is bound by an enzyme which degrades it. Such a mimotope can bind to the receptor site of the enzyme, competitively inhibiting the binding of Activin or Inhibin (as appropriate) to the enzyme and thereby inhibiting its degradation.

It may be an antagonist of an antagonist of Activin or Inhibin. For example, it may be an antagonist of Follistatin.

Activin is a member of the TGFβ superfamily, and like the other members of this family, activins are dimeric proteins, composed of disulphide linked beta A or beta B subunits. Three different forms of Activin have been identified *in vivo*: Activin A (beta a, beta a), Activin B (beta b, beta b) and Activin AB (beta a, beta b). Herein, by "Activin" is meant all possible forms of activin. Inhibins are heterodimers of beta a or beta b chains together with a common alpha chain and are called Inhibin A (alpha beta a) and Inhibin B (alpha beta b). Herein, by "Inhibin" is meant all possible forms of inhibin (Massague, J., 1990, "The Transforming Growth Factor Beta Family", Annual Review of Cellular Biochemistry, 6: 587-641. Vale, W. *et al.*, 1990, "The Inhibin /Activin Family of Hormones and Growth Factors" in Peptide Growth Factors and Their Receptors, Volume II, M.B. Sporn and A.B. Roberts (eds), Springer-Verlag, pages 211-248).

The biological response to Activins or Inhibins is transduced by receptors which exist as heteromeric complexes of type 1 receptors (called Activin receptor like

kinases (Alk) 2 and 4) and type 2 receptors which are transmembrane serine threonine kinases (Matthews, L.S. and Vale, W.W., 1993, "Molecular and Functional Characterisation of Activin Receptors", Receptor Volume 3, pages 173-181). Follistatin is an Activin binding protein which acts as an Activin antagonist *in vitro*, but *in vivo* may present Activins to their receptors (Michael, U. *et al.*, 1993, "Follistatins: more than follicle stimulating hormone suppressing proteins", Molecular and Cellular Endocrinology, Volume 91, pages 1-11).

Activin increases the number of gonadotrophs in the pituitary and causes differentiation of ovarian granulosa cells (May, K.E., 1994, "Inhibin and Activin: Molecular Aspects of Regulation and Function", TEM 5: 407-415). Activin A also enhances the differentiation or neuronal cells (Schubert, D. *et al.*, 1990, "Activin is a nerve cell survival molecule", Nature, 344: 868-870), stimulates differentiation of megakaryocytes and erythroid cells (Nishimura, M. *et al.*, 1991, "Effect of erythroid differentiation factor on megakaryocytic differentiation of L8057, a murine megakaryoblastic leukemia cell line", Biochem Biophysics Research Communication, 181: 1042-1047) and induces mesoderm formation during early Xenopus development (Smith, J.C. *et al.*, 1990, "Identification of a potent Xenopus mesoderm inducing factor as a homologue of Activin A", Nature, 345: 729-731).

Targeted disruption of the Activin beta A chain resulted in mice with craniofacial defects which died within 24 hours after birth (Matzuk, M.M. *et al.*, 1995, "Functional analysis of activins during mammalian development", Nature, 274: 354-356). These mice also lacked whiskers and had abnormal whisker follicles. Activin beta A chain has been detected in the mesenchyme of developing hair follicles and embryonic

skin, but not new born or adult skin (Roberts, V.J. *et al.*, 1991, "Expression of Inhibin/Activin sub-unit messenger ribonucleic acids during rat embryogenesis", Endocrinology 128: 3122-3129; Roberts, V.J. and Barth, S.L., 1994, "Expression of messenger ribonucleic acids encoding the Inhibin/Activin system during mid and late gestation rat embryogenesis", Endocrinology, 134: 914-923), in addition to the activin receptors Alk2 and Alk4 (Verschueren, K. *et al.*, 1995, "Expression of type 1 and type 1B receptors for activin in mid-gestation mouse embryos suggests distinct functions in organogenesis", Mechanisms of Development, 52: 109-123). Disruption of the activin-binding protein, follistatin, in transgenic mice results in abnormal whisker development and hyperkeratotic skin. (Matzuk, M.M. *et al.*, 1995, "Multiple defects and perinatal death in mice deficient in follistatin", Nature, 374: 360-363). Disruption of the gene for the Activin/Inhibin beta b subunit resulted in subtle defects to eyelid development (Vassaiil, A. *et al.*, 1994, "Activin/Inhibin beta b subunit chain disruption leads to defects in eyelid development and female reproduction", Genes and Development, 8: 414-427), whilst targeted disruption of the Inhibin alpha chain caused tumour formation in the gonads (Matzuk, M.M. *et al.*, 1992, "Inhibin is a tumour suppressor gene with gonadal specificity in mice", Nature, 360: 313-319).

There have been no reports of the role of either Activin, Inhibin or follistatin during wound healing, scarring or fibrosis.

However, the present inventor has found that Activin and Inhibin in fact play roles in wound healing as non-fibrotic growth factors. High levels of expression of Activin and of Activin and Inhibin receptors have been found post-wounding at wound sites, similar to TGF-β_3 (see PCT/GB93/00586). This observation is particularly

surprising in light of the prior belief that Activin and Inhibin are predominantly reproductive /erythroid /neurological /mesoderm inducing factors.

Activin and Inhibin have been found to be structurally similar to TGF-β_3, the similarity being greater than that with TGF-β_1 and TGF-β_2. It appears that Activin and Inhibin may in fact bind to similar receptors as TGF-β_3 and as such mediate the control of scarring *via* that route.

It has also been found that the Act 2a receptor, which is bound by Activin and which is believed to be bound by TGF-β_3, is upregulated in wound healing, especially on day 7 post-wounding. Table 1 details further the binding of the isoforms of the TGF-β receptor family.

Hence Activin and Inhibin have similar anti-scarring properties to those of TGF-β_3 and as such Activin and Inhibin may be used to similar effect (see, for example, PCT/GB93/00586).

The composition may be used in conjunction with a pharmaceutically acceptable carrier, diluent or excipient.

The composition may be used in conjunction with a composition for promoting the healing of wounds or fibrotic disorders with reduced scarring.

The composition may be used in conjunction with a composition for promoting the healing of chronic wounds.

Also provided according to the present invention is a method for promoting the healing of wounds or fibrotic disorders with reduced scarring comprising stimulating Activin and/or Inhibin.

The stimulation may be achieved by administering to a site activin and/or inhibin itself or a stimulator of Activin and/or Inhibin. By 'site' is meant a site of wounding or fibrotic disorder. The stimulator may be a stimulator or stimulating composition according to the present invention. It may, for example, be an antagonist of Follistatin.

Activin and/or Inhibin may be stimulated immediately prior to wounding. It may be stimulated immediately after wounding, although it may be stimulated later, for example within approximately 3 or 7 days or longer after wounding.

The method may be used in conjunction with a composition or method for promoting the healing of wounds or fibrotic disorders with reduced scarring.

The method may be used in conjunction with a composition or method for promoting the healing of chronic wounds.

EXPERIMENTAL

Wounding

Adult male CD1 mice were anaesthetized using halothane nitrous oxide and oxygen. Four wounds were placed on each animal, approximately one centimetre from the mid line, 20 and 40 centimetres from the base of the skull respectively. The wounds were 1 centimetre in length down to and through the paniculous carnosis. Animals were killed and wounds recovered on days 1, 3, 7, 14, 28, 60 and 80, post wounding. At least 4 wounds from 4 separate animals were analysed for each experiment. Wounds were excised, fixed in paraformaldehyde, dehydrated and embedded in wax in preparation for *in situ* hybridisation (under RNAase free conditions), or frozen in OCT (Miles Scientific), cryosectioned and utilized for immunocytochemistry.

For *in situ* hybridisation, antisense riboprobes were constructed against the Act 2a receptor, Act R1 (Alk 2) and Act RIB (Alk 4).

For immunocytochemistry, a primary antibody recognising activin was used and detected using streptavadin biotin amplification using an FITC (fluorescein isothiocyanate) labelled secondary antibody.

As controls, non wounded adult and fetal E16 (embryonic day 16) skin were used.

Results

On days 3 and 7 post wounding, enhanced staining for Activin was detected in the wound site, predominantly in fibroblasts of the wound margin and granulation tissue. Staining had returned to near normal levels by 14 days post wounding. As the antibody predominantly recognises the Activin beta A chain, it is assumed that this is the predominant isoform in the granulation tissue.

The messenger RNA for the Act 2a receptor was up-regulated in the wound margin and granulation tissue on seven days post wounding. The Alk 2 (Act R1) receptor was expressed in the mesenchyme of normal skin, but no significant elevation was detected in the wound edge or granulation tissue. By contrast, Act R1B (Alk 4) receptor was present at a much lower level in the normal skin dermis but was up-regulated in the dermal wound margin and granulation tissue of the wounds, particularly on days 7 and 14, post wounding.

In normal adult mouse skin, Alk 2 and Alk4 were expressed predominantly in the dermis and epidermis, respectively. Staining for Activin in the normal adult skin was at a marked low level in the dermis. However, fetal skin from embryonic day 16 mice showed marked staining for activin, particularly in the fetal dermis.

These staining patterns suggest that Activin and its receptors are present in fetal skin and reinduced during wound healing in adult skin. As fetal wounds heal without scarring at embryonic day 16 (Whitby, D.J. and Ferguson, M.W.J., "The extracellular matrix of lip wounds in fetal, neonatal and adult mice", Development, 112: 651-668, 1991) and with reduced levels of inflammation, and hence TGFβ1 and TGFβ2,

but enhanced endogenous dermal levels of TGFβ3 (Whitby, D.J. and Ferguson, M.W.J., 1991, "Immunohistochemical localisation of growth factors and fetal wound healing", Developmental Biology, 147: 207-215), it might reasonably be assumed that Activin plays a role in this scarless fetal wound healing. Hence, exogenous addition of Activin, or its related molecule Inhibin (which binds to similar receptors to Activin) or antagonism of the binding protein of Activin (Follistatin) could have anti-scarring activity.

Table 1 - the TGF-β Receptor family and their known affinities for TGF-$\beta_{1, 2 \text{ and } 3}$, Activin, BMP2,4 and MIS

Type I Receptors	TGF-β	Activin	BMP 2,4	MIS
TGF-β RI	✓			
Act R-Iβ		✓		
Atr-I		✓		
BRK-I			✓	
RPK-I			✓	
Act R-I		✓		
TSR-I	✓	✓		
Brk-43E			✓	
Brk-25D			✓	
DAF-I				
Type II Receptors				
Act R-II		✓		
Act R-IIB		✓		
Atr II		✓		
TGF-β RII	✓			
Daf 4			✓	
C14				?

BMP2,4 = Bone Morphogenetic Proteins

MIS = Mullerian Inhibiting Substance

CLAIMS

1. A composition for promoting the healing of wounds and fibrotic disorders with reduced scarring comprising a stimulator of Activin and/or Inhibin

2. A composition according to claim, 1 wherein the stimulator of Activin and/or Inhibin is selected from any one of the group of Activin and/or Inhibin or a fragment or an analogue thereof, an inhibitor of metabolism, and a stimulator of synthesis thereof.

3. A composition according to claim 2 wherein the stimulator of Activin and/or Inhibin comprises an analogue having a longer half-life than Activin and/or Inhibin.

4. A composition according to either one of claims 1 or 2 wherein the stimulator of Activin and/or Inhibin comprises an antagonist of an antagonist of Activin and/or Inhibin.

5. A composition according to claim 4 wherein the stimulator comprises an antagonist of Follistatin.

6. A composition according to any one of the preceding claims wherein it also comprises a pharmaceutically acceptable carrier, diluent or excipient.

7. A composition according to any one of the preceding claims when used in conjunction with a composition for promoting the healing of wounds or fibrotic disorders with reduced scarring.

8. A composition according to any one of the preceding claims when used in conjunction with a composition for promoting the healing of chronic wounds.

9. A method for promoting the healing of wounds or fibrotic disorders with reduced scarring comprising stimulating Activin and/or Inhibin.

10. A method according to claim 9 wherein the stimulation is achieved by administering to a site a stimulator of Activin and/or Inhibin.

11. A method according to claim 10 wherein the stimulator comprises a stimulator or stimulating composition according to any one of claims 1-6.

12. A method according to any one of claims 9-11 when wherein the Activin and/or Inhibin is stimulated immediately prior to wounding.

13. A method according to any one of claims 9-12 wherein the Activin and/or Inhibin is stimulated immediately after wounding.

14. A method according to any one of claims 9-13 when used in conjunction with a composition or method for promoting the healing of wounds or fibrotic disorders with reduced scarring.

15. A method according to any one of claims 9-13 when used in conjunction with a composition or method for promoting the healing of chronic wounds.

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Patents Act 1977 Examiner's report to the Comptroller under Section 17 (The Search report)	Application number GB 9521608.1
Relevant Technical Fields (i) UK Cl (Ed.O) C3H (HK1, HK2); A5B (BHA, BJA) (ii) Int Cl (Ed.6) A61K 38/18, 38/22	Search Examiner COLIN SHERRINGTON Date of completion of Search 22 JANUARY 1996
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE: EMBASE, IPA, MEDLINE, DIOGENES, CA SEARCH, BIOSIS, AGRICOLA, CABA, LSC, CEABA, DBA, WPI, CLAIMS	Documents considered relevant following a search in respect of Claims :- 1-6

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages		Relevant to claim(s)
X	EP 0617966 A1	(PERRING, SUSAN P) whole document	1-3, 6
X	WO 89/11862 A1	(BIOTECHNOLOGY AUSTRALIA PTY LTD ET AL) whole document, especially page 17, line 22 to page 18, line 4; page 18, line 9 to page 19, line 38; Claims 9, 12, 14, 15	1-3, 6
X	WO 91/12334 A1	(CETUS CORPORATION) especially Example VI; Claims 13-15, 17, 18	1, 2, 6
X	WO 92/04913 A1	(CHILDREN'S HOSPITAL MEDICAL CENTER OF NORTHERN CALIFORNIA) whole document	1-3, 6

Databases:The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).

Patents Act 1977
Examiner's report to the Comptroller under Section 17
(The Search report)

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Category	Identity of document and relevant passages		Relevant to claim(s)
X	US 5071834	(GENENTECH INC) especially column 3, line 22 to column 6, line 30; Example II; Claims 3-6	1-3, 6
X	US 5102868	(GENENTECH INC) whole document	1, 2, 6
X	Derwent WPI Abstract Accession No 95-243599/32 & JP 070149659 A		1, 2, 6
X	Arkh. Patol 1988, 50(9), 34 The effect of immunoregulatory drugs on promotion of purulent wound healing		1, 2, 6
X	US 5216004	(CHILDREN'S HOSPITAL MEDICAL CENTER OF NORTH CALIFORNIA) especially column 1, lines 61-67; column 2, lines 35-53; column 3, line 58 - column 6, line 23	1-3, 6
X	US 5428011	(PROCYON BIOPHARMA INC) whole document	1, 2, 6
X	US 5413989	(CELTRIX PHARMACEUTICALS INC) whole document	1, 2, 6